2.0 Geochemistry

2.1 Microbial Degradation

Re-evaluation of Microbial Gas Generation Under Expected Waste Isolation Pilot Plant Conditions[1]

Data Summary and Progress Report (July 14, 2001 – January 31, 2002)
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Abstract

Gas generation from the microbial degradation of the organic constituents of transuranic waste under conditions expected at the WIPP repository is being investigated at Brookhaven National Laboratory. This report summarizes progress from the period July 14, 2001 – January 31, 2002. During this period, we analyzed the total gas, carbon dioxide, and methane production; microbial degradation products of cellulose; and the diversity of microbial community in samples incubated under inundated conditions for 9.5 years (3464 days). The total volume of gas produced has leveled-off or diminished in anaerobic inundated cellulose samples with and without bentonite since the last analysis at 2718 days. Carbon dioxide production generally followed the same trend except in amended inoculated samples (2.10 μmol g^{-1} cellulose produced over 2 years (0.0028 μmol g^{-1} cellulose day^{-1})). There was a large variation in CO_2 produced between triplicate samples in the anaerobic amended inoculated samples containing excess nitrate, indicating that certain treatments may be showing further activity at 3464 days. The enhanced CO_2 production due to the addition of bentonite remains evident at 3464 days even though there was no increase in production since 2718 days. Almost all of the initially aerobic samples showed slight increases in total gas produced at 3464 days; CO_2 increased slightly in amended inoculated samples (0.0066 μmol g^{-1} cellulose day^{-1}). In general, CO_2 production in initially aerobic samples continued to remain at the same level. Up to 5.9 nmol methane g^{-1} cellulose was detected in anaerobic unamended inoculated samples, and for the first time, methane was detected in samples that were treated with excess nitrate. The rate of methane production in anaerobic samples was 2.5 pmol CH_4 g^{-1} cellulose d^{-1} in both unamended inoculated and amended treatments, and 3.45 pmol CH_4 g^{-1} cellulose d^{-1} in samples containing excess nitrate. Methane was also detected for the first time in initially aerobic inundated samples. In general, unamended samples showed the greatest quantities of methane. Acetic and butyric acids accumulated to a significant extent in anaerobic samples amended with nutrients (6.99 and 6.38 mM respectively); the accumulation of these acids was stimulated by the presence of bentonite (38.6 and 49.8 mM respectively). The overall organic acid content was lowest in anaerobic samples containing excess nitrate and in samples incubated under initially aerobic conditions. These results are consistent with oxidative processes being dominant in these samples.

[1] This work is covered by BOE #1.3.5.4.1 and WBS #1.3.5.4.1.1

Microbial characterization of anaerobic inundated samples showed *Halobacter utahensis* as the predominant species in unamended samples with the nutrient amended samples dominated by *Halobacterium*, *Halobacter*, *Halococcus*, and the haloalkalophiles *Natranobacterium* and *Natranomonas*. Work in progress includes analysis of data from studies of methane produced in humid samples and samples containing plastic and rubber materials.

BNL Project Objectives

1. Re-evaluate the existing microbial gas data and develop appropriate technical approaches to reducing the conservatism in the current gas generation model.

2. Re-examination and improvement of the experiment for cellulose degradation under humid conditions to derive a more realistic rate for humid microbial degradation.

3. Determine the effect of MgO on the rate and extent of gas generation under humid conditions.

4. Scoping experiments to clarify the factors that caused a diminishing microbial gas generation rate with time in the ongoing experiments, including testing the effect of crystallinity on cellulose degradation under hypersaline conditions.

5. Determine the rate and extent of methanogenesis by halophilic microorganisms. Due to the fact that methanogenesis is the terminal electron-accepting process in any system it is important to understand the occurrence and rate of this process.

Progress Report

Long-term experiments designed to examine gas generation due to biodegradation of the organic fraction of transuranic wastes under WIPP repository-relevant conditions have been ongoing at Brookhaven National Laboratory (BNL). Table 1 provides information about the status of these studies as of January 31, 2002.

Table 1. Status of Microbial Gas Generation Experiments at BNL.

Experiment	Start Date	Most Recent Analyses (Days/Years)*	Data Reported (this report) (Days/Years)
Long-Term Inundated Cellulose	1/29/92	*(3462 / 9.5) (CH_4 partial data set)* (2718 / 7.4) (CO_2)	(3462 / 9.5) (CH_4 complete data set) (3464 / 9.5) (CO_2) aqueous metabolite analysis (3561 / 9.9)
Initially Aerobic Humid Cellulose	4/7/93	*(3009 / 8.2)*	(methane completed, CO_2 planned for FY02)
Anaerobic Humid Cellulose	5/4/94	*(2616 / 7.2)*	(methane completed, CO_2 planned for FY02)
Inundated PE, PVC, and Neoprene	3/9/93	(2612 / 7.2)	(methane completed)
Inundated Hypalon	8/3/93	(2464 / 6.8)	(methane completed)

* Data from incubation times in italics were reported in the July 13, 2001 progress report.

Research performed during this reporting period was conducted according to Sandia National Laboratories (SNL) Waste Isolation Pilot Plant Test Plan TP-99-01, effective 3/21/01, under contract AT-8739. Note that the Sandia-approved BNL QA Program remained in effect during this reporting period, and is effectively implemented to date. During this period (July 14, 2001 – January 31, 2002) the following tasks were completed:

1. Total gas, carbon dioxide (CO_2), and methane (CH_4) analysis for samples from the long-term inundated cellulose biodegradation experiment.
2. Aqueous metabolites (organic acids and alcohols) produced due to biodegradation of cellulose in inundated samples analyzed by high-performance liquid chromatography.
3. Microbiological characterization, based upon molecular (ribosomal DNA) analyses for select samples from the long-term inundated experiment and initiated for additional samples and those from the humid experiments.
4. A protocol for the examination of gas production under conditions of MgO-constrained water activity was prepared and new experiments initiated.

More complete details of the progress made during this reporting period are provided below.

1. Quality Assurance Program: The program was approved by the SNL QA Team Lead on February 23, 2001. An audit was performed on June 6-7, 2001 to verify effective implementation, and no deficiencies that needed remedy after audit close-out were identified. This program remains in effect to date.

2. Gas Analysis of Inundated Samples: Tables 1-2 provide data for total gas and CO_2 produced in inundated cellulose samples incubated for 3462 days (the data for 1228 and 2718 days are included for comparison). Data have been corrected for gas produced in the absence of cellulose; data, reported as gas produced g^{-1} cellulose, are the mean ± SEM of analysis of triplicate samples.

Table 1. Total gas and carbon dioxide produced in anaerobic inundated cellulose samples over the three latest time periods of analysis.

Sample	Total Gas (ml g^{-1} cellulose)			Carbon Dioxide (μmol g^{-1} cellulose)		
	1228 Days	2718 Days	3464 Days	1228 Days	2718 Days	3464 Days
Anaerobic						
Unamended/ Uninoculated	-0.24 ± 0.05	-0.30 ± 0.08	-0.97 ± 0.23	3.13 ± 0.02	8.29 ± 3.77	4.56 ± 0.26
Unamended/ Inoculated	2.23 ± 12	2.45 ± 0.27	1.56 ± 0.26	13.9 ± 1.0	24.0 ± 1.7	26.1 ± 2.2
Amended/ Inoculated	3.78 ± 0.09	4.21 ± 0.04	2.72 ± 0.11	49.2 ± 0.8	66.9 ± 1.1	55.4 ± 2.6
Amended/Inoc. + Exc. Nitrate	12.12 ± 0.44	11.03 ± 0.43	9.98 ± 0.52	194 ± 4	251 ± 5	236 ± 42
Anaerobic + Bentonite						
Unamended/ Uninoculated	0.00 ± 0.04	0.26 ± 0.06	0.23 ± 0.05	4.70 ± 4.90	8.72 ± 0.55	8.05 ± 4.49
Unamended/ Inoculated	2.39 ± 0.20	2.48 ± 0.31	2.08 ± 0.68	55.2 ± 1.4	59.0 ± 7.1	58.6 ± 3.2
Amended/ Inoculated	3.62 ± 0.56	3.72 ± 0.63	2.72 ± 0.11	99.4 ± 4.4	83.6 ± 8.2	76.7 ± 3.0
Amended/Inoc. + Exc. Nitrate	14.9 ± 0.6	12.0 ± 0.4	11.1 ± 0.35	370 ± 14	350 ± 36	325 ± 14

Table 2. Total gas and carbon dioxide produced in initially aerobic inundated cellulose samples over the three latest time periods of analysis.

Sample	Total Gas (ml g^{-1} cellulose)			Carbon Dioxide (μmol g^{-1} cellulose)		
	1228 Days	2718 Days	3464 Days	1228 Days	2718 Days	3464 Days
Initially Aerobic						
Unamended/ Uninoculated	-0.04 ± 0.08	-0.02 ± 0.00	0.064 ± 0.01	4.43 ± 0.06	4.61 ± 0.14	4.70 ± 0.16
Unamended/ Inoculated	0.30 ± 0.07	0.64 ± 0.04	0.71 ± 0.04	14.4 ± 0.1	16.2 ± 0.1	12.9 ± 0.5
Amended/ Inoculated	1.42 ± 0.28	1.33 ± 0.56	1.71 ± 1.03	22.0 ± 2.9	21.9 ± 2.1	26.8 ± 3.5
Amended/Inoc. + Exc. Nitrate	10.3 ± 1.5	8.42 ± 1.40	7.15	186 ± 8	165 ± 44	150 ± 44
Initially Aerobic + Bentonite						
Unamended/ Uninoculated	0.33 ± 0.13	-0.97 ± 0.26	1.94 ± 0.21	11.0 ± 0.2	11.7 ± 0.8	5.23 ± 0.19
Unamended/ Inoculated	1.47 ± 0.22	-0.09 ± 0.04	2.72 ± 1.43	69.6 ± 4.8	73.9 ± 14.7	77.9 ± 13.1
Amended/ Inoculated	6.09 ± 0.04	4.02	2.00 ± 0.50	169 ± 11	120 ± 6	101 ± 11
Amended/Inoc. + Exc. Nitrate	8.10 ± 0.75	7.76 ± 2.34	9.08 ± 1.37	154 ± 7	233 ± 5	226 ± 56

A summary of these data is provided in the following figures: i) Figures 1 and 2 present total gas and CO_2, respectively, produced in anaerobic inundated cellulose samples over the course of the experiment, and ii) Figures 3 and 4 present the same data for initially aerobic samples. Figure 1 and Table 1 show that the total volume of gas produced has leveled-off or diminished in anaerobic samples with and without bentonite since the last analysis at 2718 days (it has been 746 days, or 2 years, between the latest and last gas analysis of these samples). Carbon dioxide production generally follows the same trend except in unamended inoculated samples (2.10 μmol g^{-1} cellulose produced over 2 years (0.0028 μmol g^{-1} cellulose day^{-1})(Table 1). There was a large variation in CO_2 produced in anaerobic amended inoculated samples containing excess nitrate (234 ± 42 μmol g^{-1} cellulose) shown in Figure 2 (error bar for open-diamond) indicating that certain treatments may be showing further activity at 3464 days (Table 1). The enhanced CO_2 production due to the addition of bentonite remains evident at 3464 days even though there was no increase in production since 2718 days. Almost all of the initially aerobic samples showed slight increases in total gas produced at 3464 days (Table 2); most significant was the unamended uninoculated samples with bentonite (0.004 ml g^{-1} cellulose day^{-1}) and amended inoculated samples (0.0005 ml g^{-1} cellulose day^{-1}). In initially aerobic samples, excess nitrate plus bentonite resulted in the production of 0.0018 ml g $cellulose^{-1}$ day^{-1} over the 2-year period since the last analysis (Figure 3). Finally, CO_2 increased slightly in initially aerobic amended inoculated samples (0.0066 μmol g^{-1} cellulose day^{-1}) and in unamended inoculated samples plus bentonite (0.005 μmol g^{-1} cellulose day^{-1}). Notwithstanding these samples, CO_2 production in initially aerobic samples has, in general, leveled off (Figure 4).

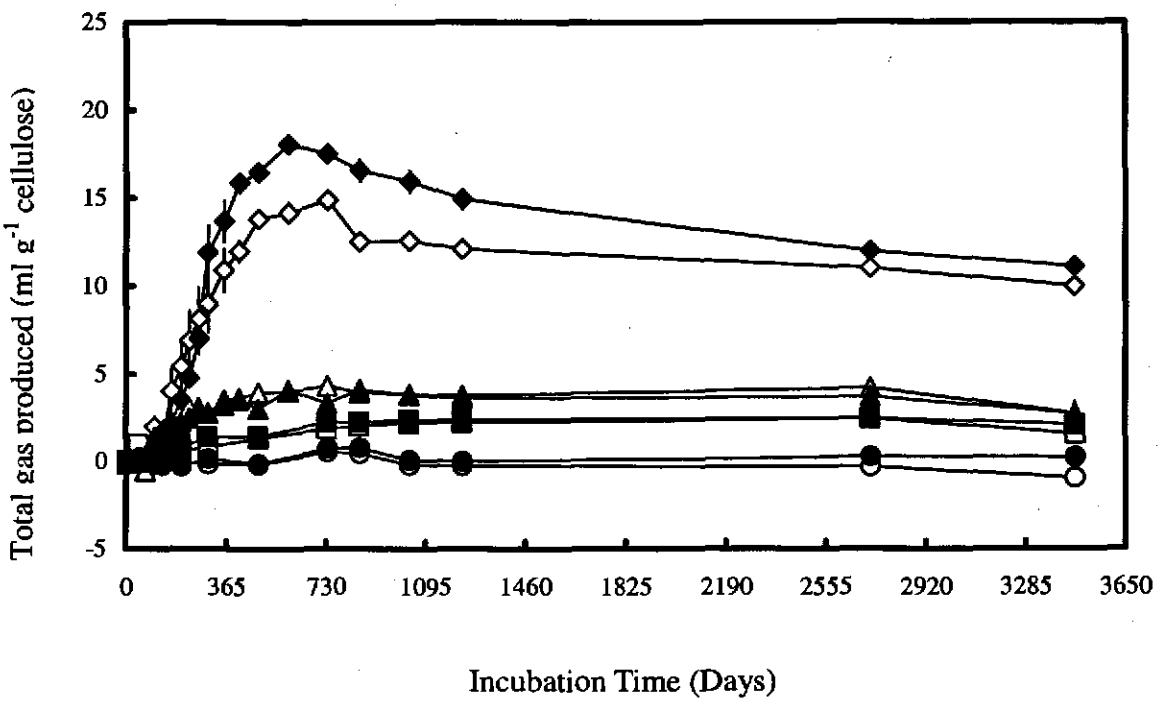

Figure 1. Total volume of gas produced in anaerobic samples inundated with brine: unamended (○); unamended and inoculated (□); amended and inoculated (△); amended, inoculated, plus excess nitrate (◊). Closed symbols are samples with bentonite.

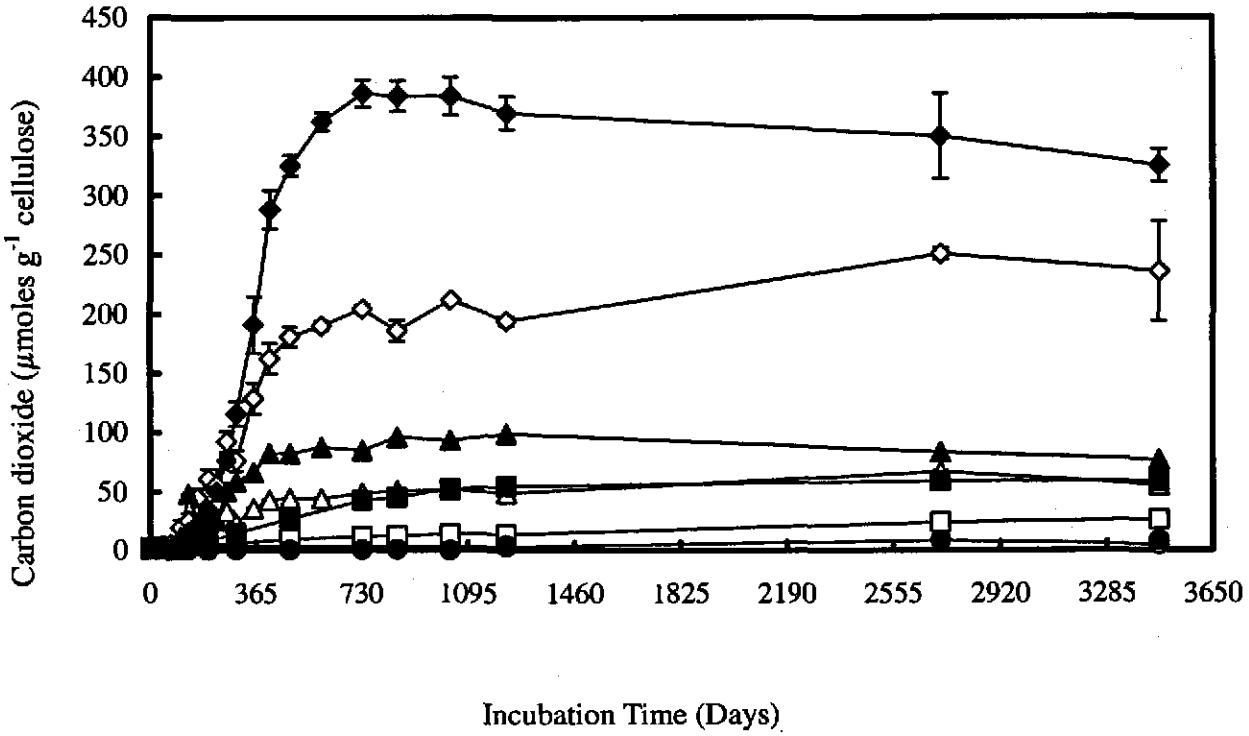

Figure 2. Carbon dioxide produced anaerobic samples inundated with brine: unamended (○); unamended and inoculated (□); amended and inoculated (△); amended, inoculated, plus excess nitrate (◊). Closed symbols are samples with bentonite.

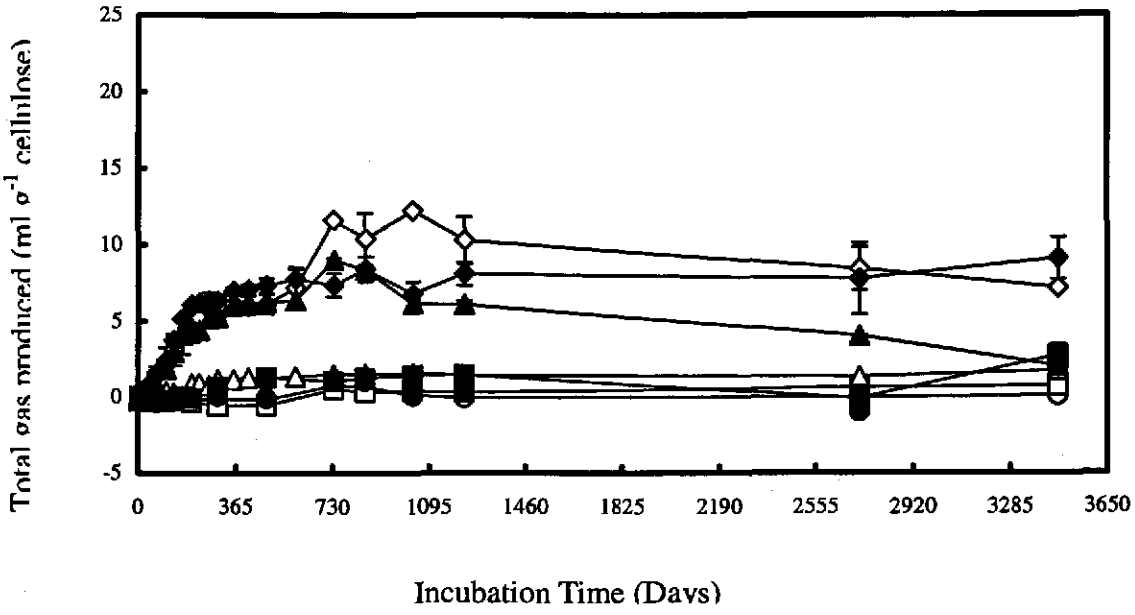

Figure 3. Total gas produced in initially aerobic samples inundated with brine: unamended (○); unamended and inoculated (□); amended and inoculated (△); amended, inoculated, plus excess nitrate (◊). Closed symbols are samples with bentonite.

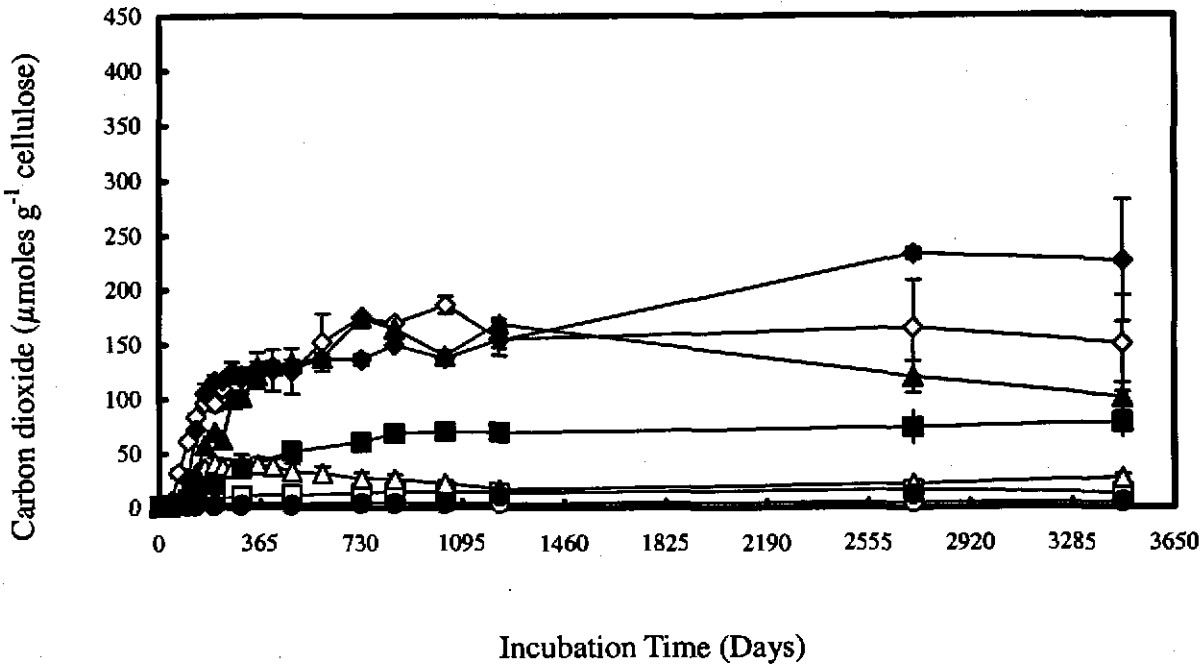

Figure 4. Carbon dioxide produced in initially aerobic samples inundated with brine: unamended (○); unamended and inoculated (□); amended and inoculated (△); amended, inoculated, plus excess nitrate (◊). Closed symbols are samples with bentonite.

3. Methane Analysis. Tables 3-4 provide data for methane analysis of inundated cellulose samples up to 3462 days incubation. Methane was analyzed by gas chromatography using flame ionization detection. The minimum detectable quantity is 0.2 nmol CH_4 g^{-1} cellulose dry wt. Methane was first detected in small quantities in most anaerobic samples except those with excess nitrate (Table 3, 2718 days). At 3462 days (9.5 years) methane was still detected in greatest quantity in samples that were not amended with any nitrogen-containing compounds (NH_4NO_3, KNO_3) specifically the unamended/inoculated samples. However, for the first time, methane has been detected in samples that initially contained excess nitrate (2.57 ± 0.79 nmol CH_4 g^{-1} cellulose (w/o bentonite) and 2.81 ± 0.16 nmol CH_4 g^{-1} cellulose (w/ bentonite). Although the time to initial production was lengthy, these samples are now accumulating methane at a relatively rapid rate: the rate of methane production was 2.5 pmol CH_4 g^{-1} cellulose d^{-1} in unamended and amended inoculated samples and 3.45 pmol CH_4 g^{-1} cellulose d^{-1} in samples containing excess nitrate (over 744 days between time 2718 and 3462). Overall, the slow rate of CH_4 accumulation, relative to CO_2, may be due to the extreme difficulty methanogens have in metabolizing the substrates such as acetate, CO_2, and H_2 (the presence of H_2 was reported in SAND96-2582, CO_2 concentrations are given in Section 2.0 of this report, and acetate concentrations are reported in the next section) under hypersaline conditions due to bioenergetic constraints (Oren, 1999). The preferred substrate is methylated amine, such as trimethylamine, commonly found in saline surface waters. Methane was detected in initially aerobic samples at 3462 days, with those samples that were not amended producing the largest initial quantities. Production rates range from 0.7 to 1.7 CH_4 pmol g^{-1} cellulose d^{-1}. Due to its importance as the terminal electron-accepting microbial process, and the need to understand production in hypersaline WIPP-relevant conditions, additional analyses of CH_4 will be performed in FY2002. Other analyses will include an attempt at subculturing the methanogens in these samples and molecular biological detection of methanogens.

Table 3. Methane analysis of anaerobic inundated cellulose samples.

Sample	Incubation Time (d) 1228	2718	3462
	---------- (nmol g^{-1} cellulose) ----------		
Anaerobic			
Unamended	nd	3.92 ± 0.27	4.40 ± 0.28
Unamended/Inoculated	nd	4.03 ± 1.38	5.89 ± 1.30
Amended/Inoculated	nd	0.85 ± 0.7	2.74 ± 0.90
Amended/Inoc. + Exc. Nitrate	nd	nd	2.57 ± 0.79
Anaerobic + Bentonite			
Unamended	nd	3.84 ± 0.40	4.51 ± 0.06
Unamended/Inoculated	nd	3.52 ± 0.20	4.06 ± 0.15
Amended/Inoculated	nd	1.12 ± 0.03	3.41 ± 0.13
Amended/Inoc. + Exc. Nitrate*	nd	nd	2.81 ± 0.16

nd = not detected

Table 4. Methane analysis of initially aerobic inundated cellulose samples.

Sample	Incubation Time (d) 1228	2718	3462
	---------- (nmol g^{-1} cellulose) ----------		
Initially Aerobic			
Unamended	nd	1.25 ± 0.29	1.82 ± 0.05
Unamended/Inoculated	nd	1.10 ± 0.13	1.34 ± 0.03
Amended/Inoculated	nd	nd	0.84 ± 0.05
Amended/Inoc. + Exc. Nitrate	nd	nd	1.27 ± 0.37
Initially Aerobic + Bentonite			
Unamended	nd	nd	1.59 ± 0.47
Unamended/Inoculated	nd	nd	2.16 ± 0.07
Amended/Inoculated	nd	nd	0.64 ± 0.06
Amended/Inoc. + Exc. Nitrate*	nd	nd	1.45 ± 0.26

nd = not detected

4. Aqueous Metabolite Analysis. Samples from the inundated experiments reserved at t=0 for aqueous chemical analysis were analyzed for organic acids and alcohols by high-performance liquid chromatography (HPLC). The presence of these aqueous metabolites, produced by bacterial metabolism of cellulose, provides insight into the effect of various nutrient treatments on the succession of microbial processes. These metabolites may accumulate and disappear depending upon microbial activity (they accumulate as a result of fermentation of glucose and they are consumed as electron-donor substrates for iron-reduction, sulfate-reduction, and methanogenesis). Finally, quantification of these metabolites provides important information relative to the carbon-balance in the samples, since cellulose hydrolysis and subsequent metabolism results in both aqueous and gaseous intermediates and end-products. A 0.25 ml sample was withdrawn from select samples and diluted to 1.0 ml with deionized water. Analytes were separated by HPLC using ion-exclusion chromatography where 1) strong and weak electrolytes (NaCl, KNO_3) are eluted unseparated at the beginning of the elution and 2) the retention times of the organic acids and alcohols are proportional to their dissociation constant values. A sulfonated macroporous styrene HPLC-column was used where analytes with higher pKa values are retained longer on the column. Acids with larger pKa values and molecular weights than butyric (pKa=4.85, MW=88.11) are separated by a secondary mechanism, hydrophobic adsorption, which is a size-exclusion phenomenon. Low-molecular weight carboxylic acids of the form $CH_3(CH_2)_nCOOH$ were principally quantified using UV detection at 210 nm; alcohols (ethanol, propanol, butanol) and glucose were quantified by refractive index detection. Retention times using both detection methods were compared to commercially-prepared standard mixtures, and both detection methods were used for positive identification of analytes. The standards included the following: 1. volatile acids (formic, acetic, propionic, butyric, isobutyric, valeric, isocaproic, isovaleric, hexanoic, heptanoic), 2. non-volatile acids (pyruvic, lactic, oxalacetic, oxalic, methyl malonic, malonic, fumaric, succinic), 3. alcohols (butanol, pentanol, propanol, ethanol), and 4. glucose. Results of HPLC of anaerobic and initially aerobic inundated samples are presented in Tables 5-8 (data are the mean of duplicate analyses; relative standard error was generally <0.5%). Previous data reported in

SAND96-2582 are included for comparison. Most of the formic and lactic acids that were produced in the early stage were consumed by 3561 days (see Table 5, unamended and amended samples). Acetic and butyric acids accumulated to a significant extent in anaerobic samples with nutrients (6.99 and 6.38 mM respectively, Table 5); the accumulation of these acids was stimulated by the presence of bentonite (38.6 and 49.8 mM respectively, Table 6). The presence of excess nitrate decreased the overall acetic and butyric acid content in anaerobic samples but formic, fumaric, and lactic acids were produced in significant quantities by 3561 days (3.26, 2.94, and 3.03 mM respectively). The accumulation of these acids at 3561 days and may indicate a shift in microbial metabolic processes which appears correlated with an increase in CO_2 production as evidenced by the large error in the data from these samples (see Figure 2 and 4, open-diamond). Bentonite, a source of iron-oxyhydroxides, stimulated the production of oxalic and oxalacetic acids (Table 6 and 8) as well as formic and fumaric acids in anaerobic samples. The organic acid content of initially aerobic samples was generally lower than anaerobic samples (Tables 7,8); this is consistent with less mature fermentative processes in these samples due to the intial bias toward aerobic respiration. Acetic acid accumulation was on par with anaerobic samples (6.91 mM, initially aerobic amended inoculated vs. 6.99 mM produced in the same anaerobic treatment and 11.0 mM initially aerobic amended inoculated plus excess nitrate vs. 5.21 mM produced in the same anaerobic treatment). The addition of bentonite resulted in decreased production of carboxylic acids in most initially aerobic samples. The production of malonic acid was unique to the initially aerobic samples. The extremely transitory nature of glucose was proven by its absence in all but one treatment (Table 6, amended inoculated samples + bentonite); the carbohydrate is consumed as fast as it is produced. Alcohols were not detected in any samples. The addition of nutrients, which stimulated microbial activity, resulted in the production of organic acids that could not be identified in certain samples; additional analyses will be performed in order to positively identify and quantify these compounds.

Table 5. Organic acids detected in anaerobic inundated cellulose samples (latest data is in bold (3561 days incubation)).

Anaerobic Treatment & Incubation Time (days)	Organic Acid (mM)								
	Acetic	Butyric	Formic	Fumaric	Lactic	Oxalic	Oxalacetic	Propionic	Succinic
Unamended									
885	0.28	nd	0.23	nd	0.05	nd	nd	nd	nd
1228	1.38	nd	1.74	nd	0.14	nd	nd	nd	nd
3561	**0.20**	**nd**	**0.13**	**0.01**	**nd**	**nd**	**nd**	**nd**	**nd**
Unamended/Inoculated									
885	1.06	nd	nd	nd	0.29	nd	nd	nd	nd
1228	3.48	nd	nd	nd	0.26	nd	nd	nd	nd
3561	**6.17**	**nd**	**nd**	**0.17**	**0.50**	**nd**	**nd**	**0.02**	**nd**
Amended/Inoculated									
885	3.73	0.16	0.48	nd	0.67	nd	nd	0.10	nd
1228	3.90	nd	1.02	nd	0.44	nd	nd	nd	nd
3561	**6.99**	**6.38**	**0.03**	**0.35**	**0.02**	**nd**	**nd**	**0.20**	**nd**
Amended/Inoculated + Excess Nitrate*									
885	nd	nd	nd	nd	nd	nd	nd	0.18	nd
1228	1.90	nd	5.95	nd	1.41	nd	nd	nd	nd
3561	**5.21**	**5.49**	**3.26**	**2.94**	**3.03**	**0.163**	**nd**	**0.43**	**nd**

*Isocaproic acid and two unknown acids with pKa, MW > butyric were detected at significant quantities at 3561 days.

Table 6. Organic acids detected in anaerobic inundated cellulose samples w/ bentonite (latest data is in bold (3561 days incubation)).

Anaerobic + Bentonite Treatment & Incubation Time (days)	Organic Acid (mM)								
	Acetic	Butyric	Formic	Fumaric	Lactic	Oxalic	Oxalacetic	Propionic	Succinic
Unamended									
885	0.20	nd	0.13	nd	0.10	nd	nd	nd	nd
1228	0.40	0.25	0.52	nd	0.06	nd	nd	nd	nd
3561	**nd**	**nd**	**0.54**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**
Unamended/Inoculated									
885	7.78	0.07	0.54	nd	2.42	nd	nd	0.17	0.30
1228	2.41	nd	0.65	nd	0.26	nd	nd	nd	nd
3561	**4.55**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**
Amended/Inoculated*									
885	6.41	0.59	0.98	nd	2.03	nd	nd	0.32	nd
1228	2.54	nd	1.80	nd	nd	nd	nd	nd	0.02
3561	**38.6**	**49.8**	**9.05**	**5.35**	**nd**	**4.04**	**0.38**	**nd**	**nd**
Amended/Inoculated + Excess Nitrate									
885	12.6	0.97	3.50	nd	20.64	nd	nd	4.52	nd
1228	8.36	1.20	15.5	nd	4.90	nd	nd	0.13	nd
3561	**8.22**	**nd**	**9.05**	**5.35**	**nd**	**nd**	**0.06**	**nd**	**nd**

*Isobutyric acid (50 mM), valeric (39 mM), glucose, and three unknown acids with pKa, MW > butyric were detected at significant quantities at 3561 days.

Table 7. Organic acids detected in initially aerobic inundated cellulose samples (latest data is in bold (3561 days incubation)).

Initially Aerobic Treatment & Incubation Time (days)	Organic Acid (mM)								
	Acetic	Butyric	Formic	Fumaric	Lactic	Oxalic	Oxalacetic	Propionic	Succinic
Unamended									
885	0.18	nd	0.39	nd	0.10	nd	nd	0.12	0.01
1228	1.30	nd	1.85	nd	0.36	nd	nd	nd	nd
3561	**0.10**	**nd**	**0.72**	**nd**	**nd**	**nd**	**nd**	**nd**	**0.01**
Unamended/Inoculated									
885	0.07	nd	0.04	nd	0.52	nd	nd	0.08	nd
1228	2.01	nd	0.87	nd	0.09	nd	nd	nd	nd
3561	**0.36**	**nd**	**0.26**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**
Amended/Inoculated*									
885	1.72	0.05	0.26	nd	1.00	nd	nd	0.30	0.52
1228	4.45	nd	2.52	nd	0.69	nd	nd	0.20	nd
3561	**6.91**	**nd**	**nd**	**1.99**	**nd**	**nd**	**0.18**	**nd**	**nd**
Amended/Inoculated + Excess Nitrate**									
885	1.23	0.09	0.33	nd	0.30	nd	nd	0.82	nd
1228	4.43	nd	3.41	nd	1.57	nd	nd	0.12	nd
3561	**11.0**	**nd**	**nd**	**nd**	**nd**	**nd**	**0.32**	**nd**	**nd**

*Malonic acid was detected at 3561 days (1.13 mM) and a significant acid (unknown) with pKa, MW>butyric.
**Malonic acid was detected at 3561 days (4.72 mM) and valeric acid (8.82 mM) as well as two acids of unknown identity (pKa > butyric).

Table 8. Organic acids detected in initially aerobic inundated cellulose samples w/ bentonite (latest data is in bold (3561 days incubation)).

Initially Aerobic + Bentonite Treatment & Incubation Time (days)	Organic Acid (mM)								
	Acetic	Butyric	Formic	Fumaric	Lactic	Oxalic	Oxalacetic	Propionic	Succinic
Unamended									
885	0.09	nd	0.16	nd	0.04	nd	nd	nd	nd
1228	1.08	nd	2.00	nd	0.10	nd	nd	nd	nd
3561	**0.13**	**0.21**	**0.63**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**
Unamended/Inoculated*									
885	3.95	nd	0.23	nd	0.86	nd	nd	nd	nd
1228	3.16	nd	2.02	nd	0.47	nd	nd	nd	nd
3561	**5.91**	**0.11**	**nd**	**nd**	**0.13**	**nd**	**nd**	**nd**	**nd**
Amended/Inoculated**									
885	4.61	0.20	0.24	nd	0.66	nd	nd	nd	nd
1228	3.66	nd	2.56	nd	1.85	nd	nd	nd	nd
3561	**7.70**	**nd**	**nd**	**nd**	**nd**	**nd**	**0.17**	**nd**	**nd**
Amended/Inoculated + Excess Nitrate***									
885	0.31	nd	nd	nd	nd	nd	nd	nd	nd
1228	1.30	nd	0.39	nd	0.06	nd	nd	nd	nd
3561	**5.00**	**nd**	**nd**	**nd**	**nd**	**nd**	**0.13**	**nd**	**nd**

*Malonic acid was detected at 3561 days (0.45 mM); ** 3561 days - malonic acid, 2.56 mM; *** 3561 days - malonic acid, 0.33 mM.

5. Microbiological Characterization: In order to completely address Objectives 1, 4, and 5, we need to understand the composition (identity and microbial community structure) of microorganisms in samples that show CO_2 and methane production. Understanding differences in community structure may help to explain difference in gas generation rates. In an anaerobic gloved box, a 10 ml sample was withdrawn from one of the triplicate reserve samples (not used for periodic gas analysis) of each of the following anaerobic inundated treatments: i) unamended, uninoculated, ii) unamended, inoculated, iii) nutrient amended and inoculated, iv) nutrient amended, inoculated, plus excess nitrate. A fifth sample, consisting of three "known" halophiles (*Halobacterium salinarium*, *Haloanaerobium praevalens*, and *Halomonas* sp.) was analyzed to verify and validate the method. Culture-independent methods were used to quantify and identify microorganisms, phospholipid fatty acid (PLFA) and denaturing gradient gel electrophoresis (DGGE) analysis, described below.

PLFA Analysis: Microbial membrane lipids, specifically phospholipid fatty acids (PLFA), were recovered from the samples according to White, et al. (1979). PLFA were analyzed by gas chromatography with peak confirmation performed by electron impact mass spectrometry (GC/MS). The individual fatty acids differ in chemical composition depending on the organism and environmental conditions. PLFA analysis provides quantitative insight into three important attributes of microbial communities: viable biomass, community structure, and metabolic activity.

DGGE: Nucleic acid extraction was performed using a bead-beating method. Polymerase chain reaction (PCR) amplification of 16S rDNA gene fragments was performed as described in Muyzer et al. (1993) with modifications. The primers targeted eubacterial 16S rDNA regions corresponding to *E. coli* positions 341-534. Purified DNA was sequenced with an ABI-Prism automatic sequencer model 377 with dye terminators. Sequence identifications were performed using the BLASTN facility of the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov/Blast) and the "Sequence Match" facility of the Ribosomal Database Project. (http://www.cme.msu.edu/RDP/analyses.html).

Results: PLFA analysis results are currently being examined and will be reported shortly, DGGE results are presented in Figure 5. Each lettered band in the figure corresponds to a unique bacterial species; the greater the number of bands the greater number of bacterial species in the samples. Higher diversity, as determined by a greater number of microbial species, was correlated with nutrient amendment and concomitant gas production. One gram-positive microorganism (genus *Clostridium,* band A, Figure 5) was detected in the anaerobic unamended uninoculated treatment; this is of interest given that almost all halophiles are gram-negative. This treatment is characterized by a low starting biomass and continual stress induced by lack of abundant electron acceptors. Introduction of mixed inoculum, but not nutrients, also resulted in dominance by one species, *Halobacter utahensis* (bands B, M, N, and O, Figure 5). In general, abundant nutrient availability lowers microbial diversity, as has been found in non-saline, low-carbon environments. Samples from the inundated cellulose experiment are analagous to environments loaded with highly complex-carbohydrates. Cellulolytic microbial populations associated with the animal rumen, a very high carbon-loading environment, have been shown to be diverse (Cho and Kim, 2000). Besides organic carbon availability, Roling et al. (2001) showed that microbial community structure in a benzene-impacted groundwater environment was determined by available electron acceptor. *Halobacterium, Haloarcula, Halobacter, and Natranobacterium* were found in the nutrient amended, inoculated treatment ((Four genera) bands C, D, E, P, Q, R, Figure 5); a fairly high diversity and unique due to the presence of *Natranobacterium.* This genus consists of species adapted to life under hypersaline, extremely alkaline conditions (pH 9-10 such as soda lakes). Excess nitrate resulted in the establishment of *Halobacterium, Halobacter, Halococcus, Natranobacterium, Natranomonas* ((Seven genera) bands F, G, H, S, T, U, and V, Figure 5), and unidentified archaea (bands S and V). The known sample resulted in the identification of three genera, thus verifying the applicability of this technique to halophilic bacteria: bands I, J (*Halomonas* sp.); K, L (*H. praevalens*); and the archaea, *H. salinarium* (band W). An obvious limitation of the technique, however, is the size of the bacterial databases; these are generally less populated with environmentally-relevant isolates, especially extremophiles, and in some instances a positive identification is not possible (eg. bands S and V, Figure 5).

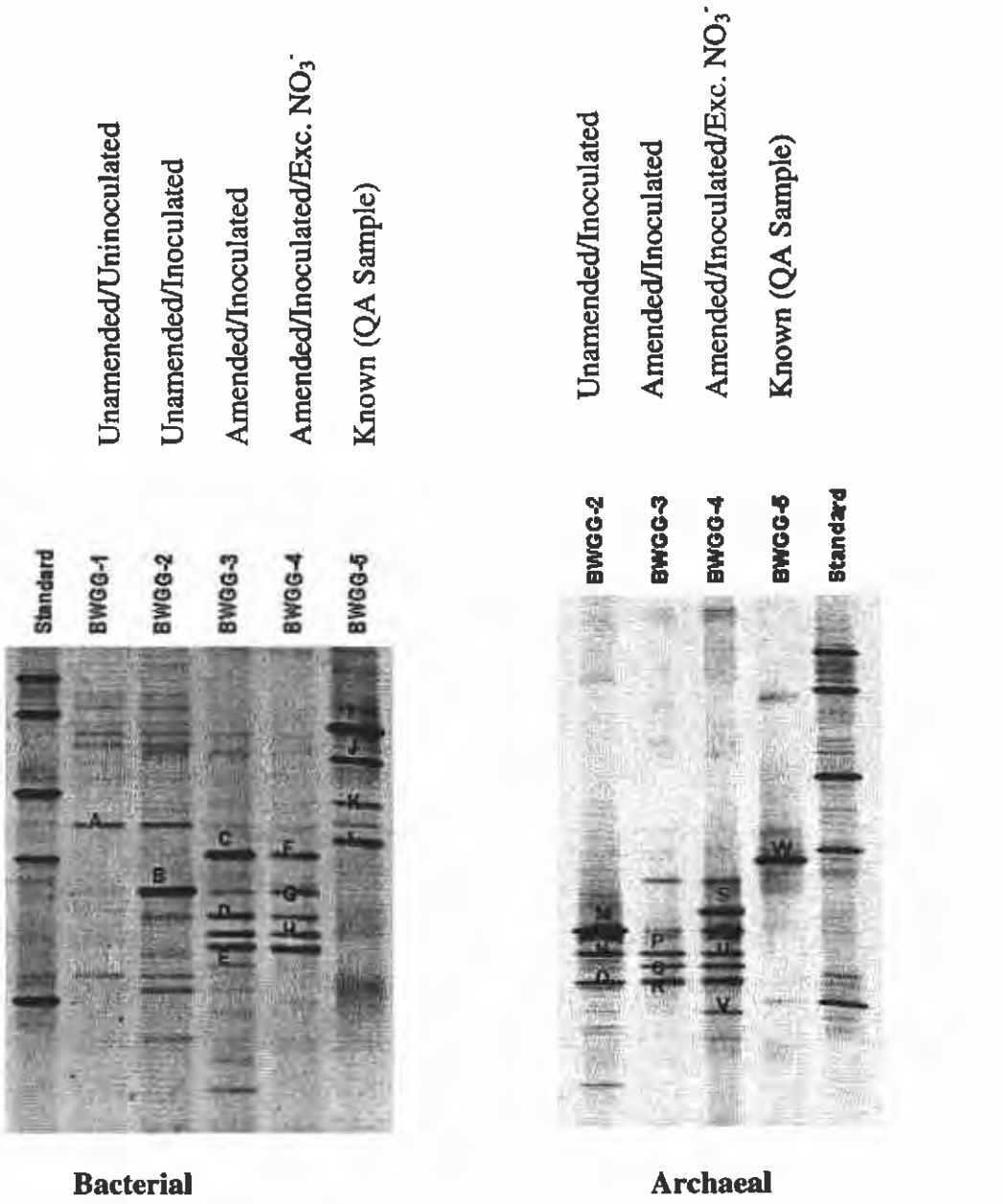

Figure 4. DGGE gel image of amplified primers from a conserved region of bacterial and archaeal 16S rDNA. Banding patterns and relative intensities of the recovered bands provide a measure of differences among the communities. Dominant species must constitute at least 1-2% of the total bacterial community to form a visible band. Labeled bands were excised and sequenced.

6. Microbial Gas Generation Under Conditions of MgO-Constrained Water Activity: The two experiments in-progress at BNL are to examine gas generation due to cellulose biodegradation under humid conditions. Samples were prepared to maintain a 70% relative humidity environment. Most of the samples in these experiments received 2.0 to 2.5 ml of liquid (2.0 ml of liquid inoculum or 2.0 ml of liquid inoculum and 0.5 ml of a nutrient solution). Experiments performed at an RH of 70% may no longer appropriately simulate WIPP disposal rooms post-closure due to the fact that MgO is being emplaced. Microbial gas generation rates under MgO-constrained humid conditions may be much lower due to sequestering of water, which is necessary for microbial activity. An experiment procedure has been prepared that tests microbial activity under more relevant MgO-constrained water activity conditions (with activity bounded at the high end by the absence of MgO and at the low-end by its presence). In order to obtain relevant gas generation data rapidly and accurately, the following will be used: i) a "dry" inoculum, ii) ^{14}C-labeled substrate (for metabolism and growth) and, iii) extremely sensitive techniques for capturing and quantifying microbially produced CO_2 (alkaline trapping and $^{14}CO_2$ liquid scintillation counting).

Progress to date: A dry inoculum was prepared by growing a *Halomonas* sp. (isolated from the WIPP environment) and its viability was confirmed. Radiolabeled substrates were obtained (^{14}C-acetate, ^{14}C-succinate, ^{14}C-cellulose) and were proven useful for providing gas-generation rates under inundated hypersaline conditions. Samples with MgO-constrained water activity were prepared and their moisture content is currently being determined by gravimetric techniques in order to standardize the treatments. A series of experiments is planned to start in early 2002.

7.0 Ongoing Work

- Select samples from the humid experiment to examine biodegradation of plastic and rubber materials have been analyzed for methane production. This data is being prepared for presentation.
- A draft manuscript, planned for submittal to a peer-reviewed journal, has been prepared that details gas production due to cellulose biodegradation under hypersaline conditions; this is currently undergoing review.
- Results of PLFA analysis of microbial populations in the anaerobic inundated cellulose samples are being examined and prepared for presentation.
- Additional samples have been taken for microbial characterization from the humid and inundated experiments; characterization work is underway.
- The experiment to examine microbial growth and gas production under conditions of MgO-constrained water activity will be started early in FY2002.

8.0 Future Work

- Aqueous chemistry data will be coupled with weight-loss determinations to provide a more complete analysis of carbon and substrate mass-balance in the samples.
- Additional aqueous chemical analyses will be performed, including i) pH, ii) further analysis of soluble carbohydrates and organic acids, iii) electron acceptors (Fe^{3+}, NO_3^-, sulfate), and iv) nitrogen/phosphorus content.
- Material characterization techniques including infrared and x-ray spectroscopy will be used to assess the extent of biopolymer degradation due to microbial activity in samples containing cellulose and plastic and rubber materials thus providing an assessment of polymer crystallinity and its relation to the extent and rate of gas generation
- A manuscript concerned with methanogenesis under hypersaline conditions will be prepared for submittal to a peer-reviewed journal.

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